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(54) METHOD FOR PRODUCING BIO-DIESEL FUEL (MONOALKYL ESTER)

(57)Abstract:
PROBLEM TO BE SOLVED: To provide a method for efficiently producing a bio-diesel fuel (bio- diesel) that can be used in conventional petroleum-based diesel engines, can be recycled and scarcely pollutes.
SOLUTION: This method for producing the bio-diesel is characterized by the esterification reaction of a fat or fatty oil with a lipase originated from a Cryptococcus genus yeast (FERM P-15155) in the presence of the fat or fatty oil and an alcohol. Thereby, the reaction can not only efficiently be carried out at one step without sequentially adding the alcohol, but the bio-diesel can efficiently be produced even when an organic solvent or water is contained.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention]It is usable, and the same engine as the diesel fuel of a petroleum base enables reproduction of this invention, and it relates to the efficient manufacturing method of biodiesel fuel with few public nuisances (it may only be hereafter called a bio diesel).
[0002]
[Description of the Prior Art]The alternative fuel with few public nuisances which emits beautiful gas came to be called for today with aggravation of the air pollution by the particles, the sulfur oxide, nitrogen oxides, and carbon monoxide which a diesel-power-plant vehicle emits. It is predicted that the energy crises by drain of fossil fuels, such as petroleum, happen on the other hand in the near future, and production of the fuel made from the raw material (biomass) in which natural reproduction like vegetable oil is possible has been an important technical problem.
[0003]The reproducible bio diesel which can use the same engine as the diesel fuel of a petroleum base in such a situation, and can reduce an exhaust gas burst size substantially has attracted attention.
[0004]A bio diesel carries out the designation of the alkyl ester produced by carrying out the ester interchange of the glycerol and alcohol in fats and oils so that it may be represented by the methyl ester (ME) of the fatty acid generated by the alcoholysis (methanolysis) of animal-and-vegetable-oils fat.
[0005]Although many of these bio diesels are produced by chemical composition now, it has many problems, such as needing a lot of energies for the difficulty of the catalyst removal after a reaction, and by-product glycerol recovery, and its manufacturing process.
[0006]Then, production of the bio diesel using lipase enzyme came to attract attention. Usually, although lipase carries out the catalyst of the reaction which hydrolyzes fats and oils (triglyceride) to fatty acid and glycerol, it is possible to make the backward reaction which carries out the ester bond of fatty acid and the alcohol perform in the conditions to which moisture was restricted. The method of carrying out the ester interchange of the lower alcohol, such as glycerol, methanol, etc. in triglyceride, by making triglyceride coexist with lower alcohol, such as methanol, and making lipase act on it is also possible.
[0007]One molecule of triglyceride needs 3-mol lower alcohol theoretically than consisting of three fatty acid and one glycerol to use 1 mol of triglyceride as a bio diesel (monoalkyl ester) thoroughly.
[0008]However, in the biodiesel production using the lipase tried until now. 1 mol of lower alcohol was made to mainly add and esterify first to 1 mol of triglyceride, and it has been performed by the training in multi stage, such as adding 1 mol of 2 more-mol alcohol at a time one by one, when the reaction is completed mostly. From the lipase generally used tending to lose activity with lower alcohol, stopping the quantity of the alcohol added at once, it adds one by one and this tries to go. In known lipase, there was a fault which poses a problem especially in industrialization that an esterification reaction is blocked in the system in which organic solvents, such as a system with a high moisture content, hexane, and petroleum ether, exist.
[0009]
[Problem(s) to be Solved by the Invention]This invention is made in order to newly

develop the manufacturing method of an efficient bio diesel (monoalkyl ester) without the above-mentioned fault.

[0010]

[Means for Solving the Problem]In [in order that this invention persons may attain the above-mentioned purpose / result of various examination] manufacture of a bio diesel, In [paying attention to enzymatic process milder than a chemosynthesis method, separate much yeast from a nature, and] a process of research on the character, Cryptococcus Espy and S-2 carry out production secretion of a lot of lipase besides enzymes, such as alpha-amylase and xylanase, and the enzyme shows strong fat-splitting nature by alkalinity from neutrality, This enzyme is stable to organic solvents, such as diethylether, chloroform, and hexane, And under coexistence with triglyceride and methanol, the ester interchange of glycerol and methanol in triglyceride was carried out, and it found out generating methyl ester of fatty acid, and generating methyl esterification of fats and oils, i.e., a bio diesel, if it puts in another way for the first time.

[0011]This invention succeeds also in manufacture of a bio diesel, and it was made based on the above-mentioned new knowledge, and it studied a physicochemical property of this enzyme and it not only checked that this enzyme was a conventionally strange new enzyme, but completes this invention at last. Hereafter, this invention is explained in full detail.

[0012]This invention performs an esterification reaction efficiently by making lipase act under existence of fats and oils (triglyceride) and alcohol, As lipase, by a single step, can carry out an ester exchange reaction by making into fundamental technical thought a point of having and manufacturing a bio diesel, and And/. Or if an ester exchange reaction can be carried out also under existence of a lot of moisture, it is also possible to use what kind of lipase. Lipase of Cryptococcus origin is illustrated as such lipase, and the physicochemical property is as follows.

[0013](1) Excel in operation fat-splitting nature, act on triglyceride, and hydrolyze into glycerin and fatty acid. the ester interchange of glycerin and alcohol in triglyceride is carried out -- if it puts in another way, the catalyst of the monoalkyl esterification of fats and oils will be carried out.

[0014](2) Substrate specificity over triglyceride and oil of substrate specificity each fatty acid is as being shown in the following table 1.

[0015]

(表1)

第1表：各種トリグリセライド及びオイルに対するリパーゼの基質特異性

基　質	＊比活性（%）
トリアセチンC_2	50.2
トリブチリンC_4	225.4
トリカプリリンC_8	269.0
トリカプリンC_{10}	56.3
トリラウリンC_2	56.3
トリミリスチンC_{14}	12.7
トリパルミチンC_{16}	125.4
トリステアリンC_{18}	18.8
トリオレイン$C_{18:1}$	100.0
オリーブ油	56.2
大豆油	60.1
米糠油	80.2
イワシ油	76.0
ココナツ油	52.0
鯨油	32.1

* % when specific activity over a triolein was set to 100 showed the specific activity of each substrate.

[0016]This enzyme decomposes tributyrin, tricaprilin, tripalmitin, and a triolein efficiently among triglyceride. On the other hand, a triacetin, a TORIKA pudding, and trilaurin had weak resolving power to a degree, a trimyristin, and a tristearin in the middle. This enzyme showed good decomposition activity to all the tested oil, and disassembled especially rice bran oil and sardine oil well.

[0017](3) The position singularity of decomposition at the time of a position singularity book enzyme acting on a triolein was investigated. By an enzyme reaction, oleic acid and a little 1,2(2,3)-Gio Reign generated, and 1,3-diolein and monoolein were not detected.

[0018](4) Optimal pH and reaction optimal pH of stable pH range refining enzymes were 7.0. High activity was shown also in pH 8.0. It was lipase which functions in the stability of pH in the range stable in pH 5 to pH 9, and wide from acescence to alkalescence.

[0019](5) The conditioned response optimal temperature of inactivation by the reaction optimal temperature and temperature was 37 **. However, inactivation of activity by a rise in heat is loose, and 71% of activity was maintained also in 60 ** and heat treatment for 30 minutes.

[0020](6) Inhibition, activation, and an organic solvent of stabilization versatility were added by 0, 2.5, 5.0, and 7.5 or 10.0% of concentration, an effect given to the activity of lipase was investigated, and a result of the following table 2 was obtained. As a result, this lipase is stable to various organic solvents except benzene, a rise of activity was rather seen depending on DMSO (dimethylsulfoxide) and diethylether, and 64.2% of activity rise was seen in DMSO7.5% of state.

[0021]
(表2)

第2表：リパーゼ活性に及ぼす有機溶媒添加の影響

溶　媒	*比活性（%）				
	0	2.5	5.0	7.5	10.0
アセトン	100	92.2	83.3	23.0	9.0
ベンゼン	100	68.6	59.7	5.3	-
クロロホルム	100	80.4	74.8	60.4	54.5
ジエチルエーテル	100	100	116.7	113.2	100.6
DMF	100	92.6	92.4	66.8	36.3
DMSO	100	108.3	121.4	164.2	124.4
ヘキサン	100	96.9	95.2	90.9	74.4
ピリジン	100	85.3	62.9	35.6	12.8
トルエン	100	80.9	71.1	55.1	34.6

* Specific activity (%) shows specific activity in various solvent concentration (%, v/v).
[0022]Thus, it is what should be mentioned specially that this lipase is stable to an organic solvent. Although, as for an ester exchange reaction and ester composition by lipase, the reaction advanced for the first time by a system in which an organic solvent and slight water are intermingled, generally it was a problem that lipase is unstable by a system in which such an organic solvent and water are intermingled.
[0023]However, also in [this lipase shows not less than 50% of activity and stability also by existence of diethylether, chloroform, and hexane, and] the bottom of existence of an organic solvent, An ester exchange reaction and ester composition are possible, therefore a bio diesel can be manufactured also from crude fats and oils in which an organic solvent used as an extracting solvent on the occasion of extraction of fats and oils remains (without refining), This enzyme fully holds activity and this enzyme is extremely excellent also in the bottom of existence of a lot of moisture from a field of new manufacture of a bio diesel, especially industrial efficient manufacture, considering these points.
[0024](7) The measuring method lipase activity of lipase activity measured p-nitorophenyl laurate (p-NPL) with the 410-nm extinction method made into a reactional substrate. Lipase activity was measured with a titrimetric method which used an olive oil as a substrate if needed.
[0025](8) Molecular weight 22kDa (SDS-PAGE)
[0026]In view of a physicochemical property which described this enzyme above, a such type enzyme was not found in lipase of the conventional known, but a new substance was presumed, and this was named lipase CS2 (in addition, this may be called CS2 lipase).
[0027]A new enzyme concerning this invention can be separated and acquired from a microorganism, for example, S-2 shares (FERM P-15155) (it may be hereafter called two shares of CS) of cultures belonging to a Cryptococcus. A strain by which invention

separation was carried out is very characteristic here at a point that the production secretion of the new enzyme (lipase CS2) can be carried out, It is a Cryptococcus about this. It was named Espy .S-2 (Cryptococcus sp. S-2) and ****ed to National Institute of Bioscience and Human-Technology, National Institute of Advanced Industrial Science and Technology, as FERM P-15155.

[0028]This enzyme can cultivate two shares of CS in accordance with a conventional method, can extract it from a culture, and can be acquired by refining. For example, let 25 ** of biomasses which carried out preculture (5.4 - 5.8x10 ^{8} KE / ml) for 40 hours be the inoculum organisms to the usual main culture in YM culture medium (0.3% of yeast extract, 0.5% of malt extract, and peptone 0.5%, and glucose 1%).

[0029]Nitrogen, a carbon source, and a thing to which an inductor was changed were made into an enzyme production culture medium if needed on the basis of 0.5% of a yeast extract, $KH_2PO_4$1%, $MgSO_4$ and 7H $_2$O 1%, and triolein 1%. What is necessary is to inoculate a bacillus which carried out preculture to a 100-ml enzyme production culture medium 1% (v/v), and just to produce an enzyme in shaking culture of 25 **100rpm. Although it is not necessary to add a triolein, production of lipase by Cryptococcus sp. S-2 An olive oil, Secretion of about 3 times as much lipase activity is seen from a glucose culture medium by making fats and oils, such as a triolein, into a carbon source, and production of lipase is raised to dominance by making fats and oils into a carbon source. It is suitable, if one is used as fats and oils as being chosen out of sardine oil, soybean oil, and triglyceride besides the above as it is few.

[0030]Although what is necessary is just to carry out culture of two shares of CS like culture of yeast, if fats and oils are used as a carbon source as mentioned above, production of an enzyme will increase. Even if a yeast extract is used as a nitrogen source and it uses lactose as sugar, production of an enzyme increases.

[0031]After cultivating two shares of CS, this enzyme is separated and refined from a culture. What is necessary is just to perform it combining known purification methods, such as chromatography treatment, after it condenses culture medium, for example although the method should just follow a conventional method. This enzyme can use enough a concentrate which obtained it by carrying out ultrafiltration of the culture supernatant, for example for manufacture of a bio diesel rather than is necessarily required for refining highly.

[0032]Thus, this obtained enzyme (CS2 lipase), Since it has the above-mentioned special feature, under existence of an organic solvent and a lot of moisture, the catalyst of an ester exchange reaction or the ester synthetic reaction can be carried out, It also has simultaneously manufacture of biodiesel fuel, and character which it not only can use especially for industrial manufacture, but usual lipase called hydrolysis of fats and oils has, and, of course, can use for medicine, cosmetics, an eating-and-drinking article, a detergent, a reagent, and other uses extensively.

[0033]In order to carry out this invention, under existence of fats and oils and alcohol with lipase. If what is necessary is just to carry out the ester interchange of glycerol and alcohol in fats and oils and lipase with necessity which is mixed, and what is necessary is just to make react, and was moreover described above while agitating is used for fats and oils, alcohol, and lipase, It is not necessary to carry out consecutive addition of the alcohol, and a reaction is performed with a step, and even if the inside of an organic solvent and a lot of moisture are intermingled in that case, higher efficacy that a reaction

advances smoothly is done so.
[0034]Thus, this invention is using lipase which yeast Cryptococcus sp. S-2 produce, It is not necessary to add 3 mol and lower alcohol beyond it, to make them react from the beginning to 1 mol of triglyceride, and to make it possible to simplify a production process of a bio diesel by lipase, is not necessary to carry out consecutive addition of the alcohol, and stands high dramatically on industry.
[0035]Generally lipase causes a reaction which hydrolyzes original triglyceride in the state with much water, and it will not become main without the state where water was restricted severely the reacting esterification which is the backward reaction. However, lipase which Cryptococcus sp. S-2 produce has the outstanding character in which the reaction advances also in a moisture content by which esterification is barred, in usual lipase. Although water will arise inevitably and an esterification reaction by lipase will be barred by accumulation of this produced water in an esterification reaction, there is no such disturbance at S-Cryptococcus sp. 2 lipase.
[0036]Although waste oil from a home used in Japan being fond as a raw material of a bio diesel also has many things containing many moisture, S-Cryptococcus sp. 2 lipase can be used as a raw material of a bio diesel, without caring about the moisture not much also in such fats and oils.
[0037]S-Cryptococcus sp. 2 lipase shows high ester productivity especially to rice bran oil also in various animal and vegetable oils. Rice bran oil is a cheap oil obtained from rice bran of rice, and can be abundantly produced in our country. It is utility that S-Cryptococcus sp. 2 lipase can produce a bio diesel efficiently especially to rice bran oil at effective use of rice bran.
[0038]Although, as for usual lipase, the activity fall is seen in existence of hexane and petroleum ether, as for S-Cryptococcus sp. 2 lipase, a rise of the activity is conversely seen by mixture of these organic solvents. Although esterification efficiency improves by mixture of hexane and petroleum ether in an esterification reaction of triglyceride and lower alcohol in S-Cryptococcus sp. 2 lipase, This shows that production of a bio diesel is rather efficiently possible by using as a raw material rough refined oil fat which removes thoroughly neither hexane used when extracting fats and oils from rice bran etc., nor petroleum ether, and is a very advantageous point on cost and a production process.
[0039]The following mode is illustrated as an embodiment of this invention.
(1) A bio diesel manufacturing method in a single step which uses lipase.
(2) Manufacture of a bio diesel by lipase in a state with many moisture contents.
(3) A bio diesel manufacturing method which uses lipase of yeast Cryptococcus sp. S-2.
(4) Bio diesel manufacture from rice bran oil.
(5) Bio diesel manufacture from hexane and crude fats and oils which various organic solvents petroleum-ether-others-mentioned already mix.
[0040]As fats and oils on which lipase is made to act, it is altogether usable in one sort of fats and oils of an animal (fishes are included) and vegetable origin, or two sorts or more, and the following are illustrated as a non-limiting example. Vegetable oil, such as olive oil, oleum rapae, soybean oil, rice bran oil, walnut oil, sesame oil, camellia oil, and peanut oil. Animal oil, such as butter, lard, beef tallow, mutton tallow, ****, and chicken oil. Fish oil, such as whale oil, sardine oil, herring oil, and cod liver oil.
[0041]Since an alkyl ester-ized reaction of fats and oils does not receive disturbance by existence of an organic solvent or a lot of moisture in this invention, even if not refined, it

is usable and usable also in a mixture of fats and oils which contain these and accompany, and these and fats and oils. [fats and oils] Therefore, solid-oils fat can also be dissolved and used for a solvent. Since this invention method is not influenced by organic solvent or moisture, it can be used suitably, without passing through a purification process with exceptional fats and oils which fats and oils manufactured by what kind of method can also be used suitably, for example, manufactured them by milling process, an extraction method, melting, etc.

[0042]As alcohol, are usable suitably in various kinds of alcohol besides lower alcohol, such as methanol, :methyl alcohol, ethyl alcohol in which the following are mentioned as the non-limiting example, Propyl alcohol, isopropyl alcohol, butyl alcohol, Isobutyl alcohol, sec-butyl alcohol, t-butyl alcohol, Fatty alcohol, such as amyl alcohol and hexyl alcohol; Allyl alcohol, Unsaturation fatty alcohol, such as propargyl alcohol; aromatic alcohol;, other various alcohol, such as alicyclic alcohol; benzyl alcohol, such as cyclohexanol and cyclopentanol, and cinnamyl alcohol.

[0043]What is necessary is to mix fats and oils, alcohol, and lipase and just to incubate, in order to carry out this invention, And although what is necessary is not to check a reaction in particular even if moisture and an organic solvent are intermingled in the system of reaction, and for there to be no exceptional restriction in a reaction condition, and just to set it as it suitably broadly from a place where it was also checked that an esterification rate, not to mention it, may go up, especially the suitable reaction condition is as follows.

[0044]the amount of enzymes -- 300-5000U -- desirable -- 500-3000U -- it being 1500-2500U still more preferably, and, as opposed to substrate weight -- quantity of water -- 5 - 500wt% -- a reaction not being checked but, even if it exists, An increase in the amount of ester is accepted under the moisture 60, not to mention it, - 100wt% of existence, When the amount of alcohol is used by a ratio of 1:1-1:4 to fats and oils, It was admitted that the one of an esterification rate where a ratio of the amount of alcohol is lower is high when there are few moisture contents (for example, when it is 50% or less), and an esterification rate of the one where a ratio of alcohol is higher was high when there are many moisture contents (for example, when it is not less than 50%).

[0045]About a reaction condition, exceptional reaction inhibition is not accepted at pH five to 9 temperature of 25-40 **, but reaction pH and reaction temperature can be chosen broadly. Although it is within the area [of selection] that reaction time also continues a reaction until the target esterification rate is attained etc. suitably, the desired end is attained as a temporary rule of thumb in about 50 to 200 hours. A reaction is good to carry out stirring at about 50-300 rpm.

[0046]this invention method Dimethyl sulfoxide (DMSO), diethylether, By existence of n-hexane, petroleum ether, and various organic solvents that were organic-solvent-others[for other benzene and fats-and-oils extraction]-mentioned already, it also has the feature that a reaction is not checked and reaction inhibition was not observed in 30% or less of case. Since a rise of an esterification rate was accepted, not to mention it, when DMSO, n-hexane, and petroleum ether were conversely added 5 to 15% depending on a kind of organic solvent, for example, it is even possible to raise an esterification rate by addition of an organic solvent. Hereafter, an example of this invention is described.

[0047]

[Example 1] Yeast extract 0.5%, lactose 0.5% and $KH_2PO_4$1%, $MgSO_4$ and 7H_2O 1%,

triolein CS2 bacillus (FERM P-15155) which carried out preculture to a culture medium for lipase production which consists of 1% was inoculated 1% (v/v), and shaking culture was carried out at initial pH5.6, 25 **, and 100 rpm for 120 hours.
[0048]Refining of an enzyme was performed as follows. 10min centrifugal separation was carried out and 8,000 rpm of culture medium except a yeast cell was condensed with an ultrafiltration after filtration with a 0.45-micrometer membrane filter. High speed liquid chromatography (gradient elution by pH 7.0 phosphoric acid buffer and a thing which carried out NaCl addition 0.5% at it) by a cation-exchange-resin TSK-gel SP-5PW column refined lipase. Activity was 17.1 times and a yield was 11.4%. It became a single band on SDS-PAGE, and, as for a molecular weight, DSS-PAGE proved that it is 22k Dalton. A physicochemical property of an obtained enzyme (CS2 lipase) was as stated above.
[0049]
[Example 2] Centrifugality of the culture medium obtained by culture 25 ** by the culture medium for lipase production (example 1) in yeast Cryptococcus sp. S-2 for 120 hours was carried out at 8,000 rpm for 20 minutes, centrifugal supernatant liquid was condensed in the 10,000 molecular-weight cut ultrafiltration membrane (YM-10) of Amicon, and enzyme liquid was obtained.
[0050]
[Example 3] A: Using CS2 lipase, methanol was made to react to fats and oils, and methyl esterification (methanolysis) of fats and oils was performed.
[0051](1) Lipase activity lipase activity was measured with the spectrophotometric analysis which used p-nitrophenyl lauric acid (p-NPL) as the substrate. The enzyme which generates p-nitrophenol of one micromole was used as enzyme activity 1 unit under the standard activity measurement method.
[0052](2) The methyl esterification oil and methanol [9.65g/0.35g (mol/mol=1/1)] mixture was used as the initial substrate. The 4-ml enzyme liquid which contains CS2 lipase of 500U in this was added within a 100-ml Erlenmeyer flask with ground-in stopper, and it was considered as the reaction mixture, and reacted by shaking at 160 rpm for 30 ** and 96 hours. The sample was isolated preparatively every 24 hours and the capillary tube gas chromatograph analyzed as follows.
[0053](3) Received the quantity of water [as opposed to 500-2,500U, and substrate weight for the amount of reaction optimization study enzymes] 13 - 200wt%, received the oil in the amount of methanol, it was made to change in 1:1-1:4, and the reaction condition was examined. Furthermore, pH five to 9 temperature of 25-40 ** examined the reaction condition. Many things were investigated also about the influence of organic solvent addition (10%, w/v).
[0054](4) The sample of 300 microliter was independently isolated preparatively from reaction mixture 3 times for every capillary tube gas chromatographic assay fixed time, and the supernatant liquid was obtained after centrifugality. In a 15-ml test tube, the TORIKA pudding of 60 microliter was made into the internal standard, 3.0 ml of hexane was added by having used anhydrous sodium sulfate as the dehydrator at 100 microliter supernatant liquid, and stirring mixing was improved. the glass chromatogram (made in Shimazu.) take the sample of 1.0 microliter from it and according to DB-5 capillary column (0.25 mmx15 m;J & W Scientific, Forsom, CA, USA) ME (methyl ester) in a reactant and FFA (the amount of free fatty acid) were measured in GC-17A. The

temperature of an injector and a detector is 245 and 250 **, respectively. After holding at 150 ** for 0.5 minute, temperature up of the column was carried out on condition of 10 ** / min after 5 ** / min, and it to 300 ** to 210 **.
[0055]B: Yeast Cryptococcus obtained in Example 2 The methyl ester of vegetable oil was performed using partially purified substance of the S-Espy .2 origin as lipase.
[0056]The result of having investigated the esterification reaction of olive oil, oleum rapae, rice bran oil, soybean oil, each, and methanol using this lipase is shown in Table 1. A reaction condition is referring to the experimental method. Rice bran oil showed the esterification rate of 24.9% at 30 ** and the reaction of 96 hours, and the best result was shown. Subsequent experiments were conducted on using rice bran oil as a substrate oil from this.
[0057]
(Table 1)
Table 1: Cryptococcus of various vegetable oil The S-Espy .2 origin Esterification by lipase (a)
---------- --------- ester conversion rate (%) (b)
oil Fat . ----------. 24 hours 48 hours 72 hours 96 hours. ----------. Olive oil 7.5 9.7 12.5 16.7 Oleum rapae 9.4 14.3 17.6 21.1 Rice bran oil 9.8 16.6 20.2 24.9 Soybean oil 9.6 12.9 16.9 21.1. The ---------- (a) reaction was performed by adding 4 ml of water which contains the lipase 500U in 9.65g fats and oils and 0.35g methanol (1:1 mol/mol).
(b) The esterification rate was expressed as a ratio of the methyl esterification to the fats and oils used as a substrate.
[0058](1) The influence (it does to rice-bran-oil methyl esterification), next the lipase addition of the amount of enzymes were reacted in the range of 500U-2,000U. Like drawing 1 in a result, the esterification rate improved with the increase in the amount of enzymes, and 2,000U showed the esterification rate of 33%. This is equivalent to the maximum value of the rate of ester obtained when 1 mol of methanol is added to the fats and oils which have 3-mol fatty acid by 1 intramolecular (drawing 1).
[0059](2) On the influence (it does to rice-bran-oil methyl esterification) moisture content of 13% of moisture, and the conditions of 48 hours, a methyl ester content (ME) is 31.5%, and 94.6% methanol in reaction mixture was consumed. Although moisture followed on increasing to 30 and 50% and the amount of esterification in the 48th hour decreased to 8.9 and 16.5%, however as shown in Table 2, 96 hours afterward, the difference was almost lost to the generated amount. On the other hand, the tendency for a direction with much [respectively] 24.0 or 42.1% and the moisture to 13% of moisture and free fatty acid 96 hours after 50% to increase was seen.
[0060]
(Table 2)
Table 2: Influence which it has on esterification and fatty acid generation of moisture content (a)
----------. moisture content methyl ester content (% of the weight) Fatty acid content (% of the weight) (% of the weight). ----------. ----------. 24 hours 48 hours 72 hours 96 hours 24 hours 48 hours 72 hours 96 hours. ----------. 13 16.4 31.5 33.3 34.0 4.7 10.3 19.9 24.0 20 22.6 31.4 33.5 34.0 12.5 21.1 30.5 33.3 30 23.0 28.7 32. 4 33.3 13.5 24.2 32.4 35.4 40 23.1 26.6 30.1 33.3 18.7 31.5

38.0 40.0 50 22.5 26.3 28.5 32.4 22.5 35.4 39.1 42.1. ----------. (a) It reacted by the system of reaction which consists of various moisture contents (1.3-5.0ml=13 - 50 substrate weight %) as the mixture of the oil 9.65g and the methanol 0.35g (1:1 mol/mol), and enzyme liquid containing the lipase 2000U.

[0061](3) At least 3-mol methanol is required to change thoroughly the influence (it does to rice-bran-oil methyl esterification) fats and oils of a moisture content and a methanol addition into fatty acid ester. However, it is necessary to take the method of adding every 1 mol of methanol one by one, by an esterification reaction from inactivation of an enzyme breaking out in the system of reaction which contains methanol of 1 mol or more, for example with lipase of Rhizopus oryzae.

[0062]However, if it does not inactivate when the lipase to be used increases methanol, the amount of methanol will be increased from the beginning and it will become that it is [operation] easier to have been able to reduce the number of times of addition. Then, the ratio of methanol was reacted to fats and oils by 1:1 to 1:4, and the step method which adds the content of water from 20 at once first 200% to substrate weight, respectively. As a result was shown in drawing 2, the methanol ratio followed on increasing (1:1-1:4), and it followed on increasing in the 60 to 100% of moisture content range, and the amount of methyl ester increased (drawing 2).

[0063]It was checked that it is possible to add and to make much methanol react at once from the beginning with S-Cryptococcus sp. 2 lipase from the above thing. In common lipase, such as Pseudomonasfluorescens and Mucor miehei, when moisture was added into reaction mixture, decline in an ester conversion rate was imitated and the opposite effect that it was [a conversion rate] better for the moisture of the quantity of the grade whose ** is this lipase to exist was observed.

[0064](4) On the conditions of 80% of addition moisture that pH and the influence substrate of temperature receive, the influence which it has on the esterification reaction of pH was investigated. Although the result was shown in drawing 3, the reaction fell as pH went up from pH 5 investigated in the range of 9. However, although pH adjustment was not carried out as a result, the ester conversion with the highest direction was shown. This is considered that existence of the salt used for pH adjustment is because it works inhibitory to an enzyme reaction. It was shown that it is not necessary to carry out pH adjustment in particular as a result (drawing 3).

[0065]The influence of temperature is shown in drawing 4, although the one for a short period of time (24 hours) where temperature is lower is better of 25, 30, and 35 or 40 ** -- 96 hours -- 30 ** -- only -- foolish ** -- the high result was obtained (drawing 4).

[0066](5) From the fats and oils of 1 mol of methanol ratios comprising 3-mol fatty acid and 1 mol of glycerol, 3-mol alcohol is theoretically required of perfect esterification of fats and oils. In esterification of the fats and oils in a synthetic chemistry reaction, in order to raise esterification efficiency, the system of reaction which uses 6 mol of alcohol to 1 mol of fats and oils is also taken. Then, also in our system of reaction which uses an enzyme, it reacted by changing fats and oils and an alcoholic ratio in 1:2 to 1:6. Although the result was shown in drawing 5, compared with the time of 1:3, 11.8% of esterification rate rise (the 120th hour) was seen in 1:4. 1:4 or more effects were not seen in 1:5, but it decreased in 1:6 (drawing 5).

[0067]The above result was synthesized and it found out that conditions (80% of substrates, the lipase activity 2,000U, 120 hours, and 30 **) were one example of the best

conditions about 1:4 and moisture in the ratio of fats and oils to methanol.
[0068](6) Quantity addition of addition effect DMSO of an organic solvent, diethylether, n-hexane, and the petroleum ether was carried out 10% each, and the influence which it has on the esterification reaction of an organic solvent was seen. A result is shown in Table 3. 4.8 to 7.0% of esterification rate rise was seen by adding DMSO, n-hexane, and petroleum ether. This is organic solvents', such as n-hexane's, being used when extracting fats and oils from vegetation, but using the cheap rough refined oil fat containing some of these organic solvents as a raw material, and the merit from which ester is rather obtained at high efficiency is obtained.
[0069](Table 3)
Table 3: Influence of organic solvent (10%, w/v) on ester (a)
---------- ----- organic solvent ME content (% of the weight)
----------. Additive-free (b) 80.2DMSO 85.0 diethylether 70.4 n-hexane 86.1 petroleum ether 87.2. The reaction was performed at 30 ** for 120 hours under existence of ---------- ----- (a) oil 9.65g / methanol 1.4g (1:4 mol/mol) mixture, 8.84 ml (lipase 2000U content) of water, and a solvent.
(b) Use of the solvent was canceled, and also it is the above and reaction-of-identity conditions, and reacted.
[0070]
[Effect of the Invention]This invention enabled it to manufacture biodiesel fuel at only 1 process very efficiently. In this invention, even if an organic solvent and a lot of moisture were intermingled in the system of reaction, it used that esterification of fats and oils advanced smoothly.
This becomes possible [waste oil, such as a waste food oil, or the mixture of those] to bio-diesel-ize, it becomes unnecessary to discard these in a river etc., and this invention stands high also from the field of environmental protection as a waste-liquid-treatment method.

[Translation done.]

Claim(s)]
[Claim 1]A manufacturing method of a bio diesel performing an esterification reaction of fats and oils by a single step under existence of fats and oils and alcohol using lipase.
[Claim 2]A manufacturing method of a bio diesel performing an esterification reaction of fats and oils in spite of mixture of moisture under existence of fats and oils and alcohol using lipase.
[Claim 3]A manufacturing method of a bio diesel performing an esterification reaction of fats and oils under existence of fats and oils and alcohol using lipase of Cryptococcus (Cryptococcus) group yeast origin.
[Claim 4]Cryptococcus yeast is a Cryptococcus. A manufacturing method of the bio diesel according to claim 3 being Espy .S-2 (Cryptococcus sp. S-2) (FERM P-15155).
[Claim 5]A manufacturing method of a bio diesel using lipase CS2 which has the following physicochemical property.

(1) It has operation fat-splitting nature, act on triglyceride, and hydrolyze into glycerin and fatty acid. The catalyst of the esterification reaction of fats and oils, and the bottom of existence of alcohol and fats and oils is carried out.
(2) Decompose substrate specificity tributyrin, tricaprilin, tripalmitin, and a triolein well.
(3) If you make it act on a position singularity triolein, oleic acid and a little 1,2(2,3)-Gio Reign will generate, and 1,3-diolein and monoolein will not be detected.
(4) optimal pH and stable pH range optimal pH: -- 7.0 stable pH range: -- conditioned response optimal temperature [of inactivation by 5 - 9(5) reaction optimal temperature and temperature]: -- condition [of inactivation by 37 ** temperature]: -- inactivation of activity by a rise in heat is loose, and activity is maintained also in 60 ** and heat treatment for 30 minutes.
(6) It is stable to stability and an activation organic solvent to an organic solvent, and activity goes up by dimethyl sulfoxide and diethylether further.
[Claim 6]A method given in any 1 paragraph of claims 1-5 using vegetable oil as fats and oils.
[Claim 7]A method of using rice bran oil as vegetable oil according to claim 6.
[Claim 8]A method given in any 1 paragraph of claims 1-7 using fats and oils in which waste oil or an organic solvent is intermingled as fats and oils.